Identification of *Phytophthora* Isolates to Species Level Using Restriction Fragment Length Polymorphism Analysis of a Polymerase Chain Reaction-Amplified Region of Mitochondrial DNA

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ABSTRACT

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Polymerase chain reaction primers spanning the mitochondrially encoded *cox*I and II genes have been identified that were capable of amplifying target DNA from all 152 isolates of 31 species in the genus *Phytophthora* that were tested. Digestion of the amplicons with restriction enzymes generated species-specific restriction fragment length polymorphism banding profiles that were effective for isolate classification to a species level. Of the 24 species in which multiple isolates were examined, intraspecific polymorphisms were not observed for 16 species, while 5 species exhibited limited intraspecific polymorphism that could be explained by the addition/loss of a single restriction site. Intraspecific polymorphisms were observed for *P. megakarya*, *P. megasperma*, and *P. syringae*; however, these differences may be a reflection of the variation that exists in these species as reported in the literature. Although digestion with *Alu*I alone could differentiate most species tested, single digests with a total of four restriction enzymes were used in this investigation to enhance the accuracy of the technique and minimize the effect of intraspecific variability on correct isolate identification. The use of the computer program BioNumerics simplified data analysis and identification of isolates. Successful template amplification was obtained with DNA recovered from hyphae using a boiling miniprep procedure, thereby reducing the time and materials needed for conducting this analysis.

Phytophthora is a complex genus within the oomycetes containing approximately 67 described species that occupy a variety of terrestrial ecological habitats (18). Many of the species are economically important plant pathogens capable of causing significant losses in a multitude of crop plants. Historically, a range of morphological and physiological criteria has been used to classify members of this genus (49,54), including sporangial structure, type of antheridial attachment (amphigynous or paragynous), host specificity, and breeding system (homothallic or heterothallic). However, morphological identification can be a time-consuming process and, due to variation in some characteristics among isolates of the same species, accurate isolate identification can require substantial expertise in *Phytophthora* taxonomy.

In addition to morphological approaches, other methods have been used to simplify and improve the accuracy of identification of isolates to a species level, including use of protein patterns (3,29), isozymes (40–42,45–47), serology (2), restriction fragment length polymorphism (RFLP) analysis of nuclear and mitochondrial DNA (10,12,20,22,32,43,48,52), and more recently single-strand-conformational polymorphism (SSCP) analysis of the internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) (31). Species-specific probes and primers have proven useful in Phytophthora disease diagnosis because their of high levels of sensitivity and generally high specificity for accurate identification (17,24,25,39,44,51). Some DNA-based methods are advantageous because pathogen isolation is not required, and polymerase chain reaction (PCR) amplification can be performed directly from DNA extracted from infected tissue. This is particularly important for diagnostic labs because it enhances sample throughput and simplifies diagnosis in cases where it may be difficult to isolate the pathogen.

DNA sequence data obtained in phylogenetic studies have also been used to differentiate *Phytophthora* species. Specific regions that have been examined include the large and small subunits of the ribosomal RNA (rRNA; 8,53) and the ITS regions of the rDNA (11–13,21,23). However, there are limits to the resolving power for some closely related species with some of these regions. For example, species such as *P. infestans*, *P. mirabilis*, and *P. phaseoli* are poorly resolved with ITS sequence data and *P. fragariae* var. *fragariae* and *P. fragariae* var. *rubi* cannot be differentiated (11). While sequence analysis of the mitochondrially encoded cytochrome oxidase I and II genes (*cox*I and *cox*II) does resolve these species (37,38), this type of analysis may be too involved for routine identification of isolates.

The ability to rapidly and accurately identify isolates to a species level using molecular markers would simplify identification of unknown isolates. The objective of this study was to develop a PCR-based RFLP assay procedure that would address this need. In view of the greater degree of sequence divergence observed in some regions of the mitochondrial DNA compared with the ITS region (11,37,38), the focus of this study was the mitochondrial DNA. Prior studies identified PCR primers for amplification of an approximately 2.2-kb amplicon containing the *cox*I and II genes and the spacer region between them (37,38). The objective of this study was to evaluate the utility of RFLP analysis of this amplicon for species identification in the genus *Phytophthora*.

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*The *e*-Xtra logo stands for "electronic extra" and indicates that the online version contains supplemental material not included in the print edition. The online version includes a link to F. Martin's website where additional data is presented.

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TABLE 1. Isolates of *Phytophthora* spp. used in this investigation

Species	Group[a]	Isolate[b]	Host	Origin
P. arecae	II	IMI 34342	*Cocos nucifera*	Indonesia
P. boehmeriae	II	325^{PT}, P1257^{UCR}	*Boehmeriae nivea*	Papua New Guinea
P. cactorum	I	311^{PT}	*Pseudotsugae menziesii*	Washington
		383^{PT}, NY422^{WW}	*Fragaria ×ananassa*	New York
		384^{PT}, NY577^{WW}	*Fragaria ×ananassa*	New York
		385^{PT}, NY568^{WW}	*Malus sylvestris*	New York
		SB2067^{GB}	*Fragaria ×ananassa*	California
		SB2079^{GB}	*Fragaria ×ananassa*	California
		25-4-3^{GB}	*Fragaria ×ananassa*	California
		GB2462B^{GB}	*Fragaria ×ananassa*	California
P. capsici	II	302^{PT}(A-1 mating type)	*Capsicum annuum*	Florida
		303^{PT}		
		304^{PT} (A-1 mating type)	*Cucurbita* sp.	New Jersey
		307^{PT} (A-2 mating type)	*Capsicum annuum*	New Jersey
		Cp-1^{DJM} (A-1 mating type)	*Theobroma cacao*	Brazil
		Cp-22^{DJM} (A-1 mating type)	*Citrullus lantatus*	Florida
		Cp-25^{DJM} (A-2 mating type)	*Citrullus lantatus*	Florida
		Cp-28^{DJM} (A-2 mating type)	*Cucurbita* sp.	Florida
		Cp-30^{DJM} (A-1 mating type)	*Capsicum annuum*	Florida
		Cp-32^{DJM} (A-2 mating type)	*Capsicum annuum*	Florida
		Cp-36^{DJM} (A-2 mating type)	*Lycopersicon esculentum*	Florida
P. cinnamomi	VI	Cn-2^{DJM} (A-2 mating type)	*Vaccinium* spp.	Florida
		446^{PT}, GB32-10^{GB}	*Castanea* sp.	California
		447^{PT}, GB32-67^{GB}	*Juglans* sp.	California
		448^{PT}, GB34-90^{GB}	*Juglans* sp.	California
P. citricola	III	Cr-4^{DJM}	*Cornus* spp.	
		SB2078^{GB}	*Fragaria ×ananassa*	California
		SB2084^{GB}	*Fragaria ×ananassa*	California
P. citrophthora	II	Ct-1^{DJM}	*Theobroma cacao*	Brazil
P. colocasiae	IV	345^{PT}, ATCC 56193, P1696^{UCR}	*Colocasia esculenta*	China
		346^{PT}, P3773^{UCR}	*Colocasia esculenta*	Indonesia
		347^{PT}, ATCC 52233, P1179^{UCR}	*Colocasia esculenta*	India
		348^{PT}, P6102^{UCR}	*Colocasia esculenta*	India
		349^{PT}, P6395^{UCR}	*Colocasia esculenta*	Indonesia
		350^{PT}, P6396^{UCR}	*Colocasia esculenta*	Indonesia
P. cryptogea	VI	400^{PT}, ATCC 36301	*Solanum tuberosum*	Ohio
		438^{PT}, IMI 045168	*Lycopersicon esculentum*	New Zealand
P. drechsleri	VI	301^{PT}, 6503^{DS}	*Capsicum* spp.	Mexico
		401^{PT}, ATCC 64494, PC3^{DS}	*Solanum tuberosum*	Egypt
		ATCC 46724 (type)	*Beta vulgaris*	U.S.A.
P. erythroseptica	VI	355^{PT}	*Solanum tuberosum*	Maine
		365^{PT}	*Solanum tuberosum*	Minnesota
		366^{PT}, ATCC 36302	*Solanum tuberosum*	Ohio
		367^{PT}	*Solanum tuberosum*	New York
		368^{PT} (metalaxyl resistant)	*Solanum tuberosum*	New York
		370^{PT}	*Solanum tuberosum*	Minnesota
		374^{PT}	*Solanum tuberosum*	Maine
		387^{PT}, NY513^{WW}	*Solanum tuberosum*	California
		388^{PT}, NY559^{WW}, IMI34684	*Solanum tuberosum*	Ireland
P. fragariae var. *fragariae*	V	394^{PT}, ATCC 13973	*Fragaria ×ananassa*	Maryland
		395^{PT}, ATCC 13974	*Fragaria ×ananassa*	New York
		396^{PT}	*Fragaria ×ananassa*	Maine
		398^{PT}	*Fragaria ×ananassa*	Oregon
		399^{PT}	*Fragaria ×ananassa*	Oregon
P. fragariae var. *rubi*	V	397^{PT}	*Rubus* spp.	Australia
P. gonapodyides	IV	393^{PT}, NY353^{WW}	*Malus sylvestris*	New York
P. heveae	II	Hv-2^{DJM}	*Theobroma cacao*	Brazil
P. hibernalis	IV	337^{PT}, ATCC 32995, P0647^{UCR}	*Citrus sinensis*	California
		378^{PT}, ATCC 56353, P3822^{UCR}	*Citrus sinensis*	Australia
		379^{PT}, ATCC 64708, CBS 522.77	*Aquilegia vulgaris*	New Zealand
		380^{PT}, ATCC 60352, CBS 270.31	*Citrus sinensis*	Portugal
P. ilicis	IV	343^{PT}, P6099^{UCR}, 771^{PH}	*Ilex aquifolium*	Oregon
		344^{PT}, ATCC 56615, P3939^{UCR}	*Ilex aquifolium*	Canada
		353^{PT}, P6100^{UCR}, 802^{PH}	*Ilex aquifolium*	Oregon
P. infestans	IV	127^{PT}, ATCC 48723	*Solanum tuberosum*	New York
		176^{PT}, 915^{KD} (A-2 mating type)	*Solanum tuberosum*	Pennsylvania
		180^{PT}, WW-IX^{KD} (A-1 mating type)	*Solanum tuberosum*	Washington
		198^{PT} (A-1 mating type)	*Solanum tuberosum*	Wisconsin
		199^{PT} (A-2 mating type)	*Solanum tuberosum*	Wisconsin
		550^{PT}, ATCC 64095	*Solanum stoloniferum*	Mexico
		580^{PT}	*Solanum demissum*	Mexico
		618^{PT} (A-2 mating type)	*Solanum tuberosum*	Mexico
		800^{PT}	*Solanum tuberosum*	Peru

(*Continued on next page*)

[a] Waterhouse morphological group (54).

[b] Isolate number from different labs that have worked with the same culture; CB = Clive Brasier, GB = Greg Browne, KD = Ken Deahl, JG = Jim Graham, EH = E. Hansen, PH = Phil Hamm and E. Hansen, DJM = Dave Mitchell, DS = Dave Shaw, PT = Paul Tooley, TJ = T. Jung, UCR = *Phytophthora* species collection, University of California at Riverside, WW = Wayne Wilcox, DR = Dave Rizzo, MG = M. Garbelotto, SW = S. Werres, and CDFA = California Department of Food and Agriculture.

TABLE 1. (*Continued from preceding page*)

Species	Group[a]	Isolate[b]	Host	Origin
		1103^{PT} (A-2 mating type)	*Solanum tuberosum*	Netherlands
		1300^{PT}	*Solanum tuberosum*	Japan
P. lateralis	V	IMI 040503 (type)	*Chamaecyparis lawsoniana*	U.S.A.
		451^{PT}	*Chamaecyparis lawsoniana*	
		455^{PT}, T4P3^{EH}	*Chamaecyparis lawsoniana*	Oregon
P. megakarya	II	327^{PT}, P132^{CB}, P1668^{UCR}, IMI202535	*Theobroma cacao*	Nigeria
		328^{PT}, P184^{CB}, P1672^{UCR}, IMI202077	*Theobroma cacao*	Cameroon
P. megasperma	V	309^{PT}, 336^{PH}, 23^{EH}	*Pseudotsugae menziesii*	Washington
		335^{PT}, 63^{EH}, 261S-1^{WW}	*Prunus* spp.	California
		336^{PT}, 77^{EH}, 304^{PH}	*Pseudotsugae menziesii*	Oregon
		IMI 133317, 400^{EH}	*Malus sylvestris*	Australia
P. mirabilis	IV	340^{PT}, ATCC 64070, P3007^{UCR}	*Mirabilis jalapa*	Mexico
		341^{PT}, ATCC 64072, P3009^{UCR}	*Mirabilis jalapa*	Mexico
		342^{PT}, ATCC 64073, P3010^{UCR}	*Mirabilis jalapa*	Mexico
		354^{PT}, IMI 141668, P6917^{UCR}	*Mirabilis jalapa*	Mexico
P. nemorosa	IV	P-13^{EH}, 482^{PT} (type)	*Lithocarpus densiflorus*	California
		2052.1^{EH}, 483^{PT}	*Lithocarpus densiflorus*	Oregon
P. nicotianae	II	359^{PT}	*Solanum tuberosum*	Delaware
		361^{PT}	*Solanum tuberosum*	Delaware
		363^{PT}	*Solanum tuberosum*	Florida
		Pn-17^{DJM} (A-1 mating type)	*Citrus* spp.	Florida
		Pn-19^{DJM}	*Citrus* spp.	Florida
		Pn-21^{DJM}	*Vinca* sp.	Florida
		Pn-23^{DJM}	*Nicotiana tabacum*	Florida
		Pn-26^{DJM}	*Nicotiana tabacum*	Georgia
		331^{PT}	*Nicotiana tabacum*	North Carolina
		332^{PT}	*Nicotiana tabacum*	Australia
		333^{PT}	*Lycopersicon esculentum*	California
		334^{PT}	*Lycopersicon esculentum*	Australia
		P259^{JG}	*Liriope* sp.	Florida
		Pn198^{JG}	*Citrus* sp.	Florida
P. palmivora	II	329^{PT}, P131^{CB}	*Theobroma cacao*	Nigeria
		Pl-5^{DJM}, P626^{UCR}	*Theobroma cacao*	Brazil
		Pl-10^{DJM}	*Theobroma cacao*	Costa Rica
		Pl-14^{DJM}	*Citrus* sp.	Florida
		Pp99^{JG}	*Citrus* sp.	Florida
P. phaseoli	IV	330^{PT}	*Phaseolus lunatus*	Maryland
		352^{PT}, ATCC 60171, CBS 556.88	*Phaseolus lunatus*	unknown
		373^{PT},	*Phaseolus lunatus*	Delaware
		402^{PT}	*Phaseolus lunatus*	Delaware
		403^{PT}	*Phaseolus lunatus*	Delaware
		406^{PT} (race D)	*Phaseolus lunatus*	Maryland
P. pseudosyringae	IV	PSEU16^{TJ}, 484^{PT}, NFV-BU97-15	*Fagus sylvatica*	Germany
		P96^{EH}, 485^{PT}	*Umbellularia californica*	California
		470^{PT}, P193907A^{CDFA}	*Manzanita* sp.	California
		471^{PT}, 1168699^{CDFA}	*Umbellularia californica*	California
		472^{PT}, 1168676^{CDFA}	*Umbellularia californica*	California
		473^{PT}, P110361^{CDFA}	*Umbellularia californica*	California
P. pseudotsugae	I	308^{PT}, H270^{PH}	*Pseudotsugae menziesii*	Oregon
P. ramorum	IV	Prn-1^{PT}, PD93/844^{SW}	*Rhododendron* sp.	Netherlands
		Prn-2^{PT}, PD94/844^{SW}	*Rhododendron* sp.	Netherlands
		Prn-3^{PT}, PD98/8/6743^{SW}	*Rhododendron* sp.	Netherlands
		Prn-4^{PT}, PD98/8/6285^{SW}	*Rhododendron* sp.	Netherlands
		Prn-5^{PT}, PD98/8/2627^{SW}	*Rhododendron* sp.	Netherlands
		Prn-6^{PT}, PD98/8/5233^{SW}	*Viburnum* sp.	Netherlands
		Prg-1^{PT}, BBA 69082^{SW}	*Rhododendron* sp.	Germany
		Prg-2^{PT}, BBA 9/95^{SW}, CBS101553 (type)	*Rhododendron catawbiense*	Germany
		Prg-3^{PT}, BBA 14/98-a^{SW}	*Rhododendron catawbiense*	Germany
		Prg-4^{PT}, BBA 12/98^{SW}	*Rhododendron catawbiense*	Germany
		Prg-5^{PT}, BBA 13/99-1^{SW}	*Rhododendron catawbiense*	Germany
		Prg-6^{PT}, BBA 16/99^{SW}	*Viburnum bodnatense*	Germany
		Prg-7^{PT}, BBA 9/3^{SW}	Water	Germany
		Prg-8^{PT}, BBA 104^{SW}	Water	Germany
		288^{MG}	*Rhododendron* sp.	California
		73101^{CDFA}	*Lithocarpus densiflorus*	California
		044519^{CDFA}	*Umbellularia californica*	California
		044522^{CDFA}	*Lithocarpus densiflorus*	California
		P072648^{CDFA}	*Quercus agrifolia*	California
		201C^{DR}	*Rhododendron* sp.	California
		0217^{DR}	*Rhododendron* sp.	California
		Coen^{MG}	*Rhododendron* sp.	California
		013^{DR}	*Lithocarpus densiflorus*	California
		016^{DR}	*Quercus agrifolia*	California
P. sojae	V	312^{PT}, ATCC 48068	*Glycine max*	Wisconsin
		313^{PT}, ATCC 48069	*Glycine max*	Wisconsin
		314^{PT}, ATCC 52693	*Glycine max*	Wisconsin
P. syringae	III	IMI 296829	*Rubus idaeus*	Scotland
		468^{PT}	*Kalmia latifolia*	Oregon
		469^{PT}	*Kalmia latifolia*	Oregon

MATERIALS AND METHODS

***Phytophthora* cultures and DNA extraction.** The cultures used in this study are listed in Table 1; many of them have been reported on previously (37–39). Cultures were grown on rye A medium (9) at 20°C in darkness and maintained in liquid nitrogen for long-term storage (50). DNA was extracted by the method of Goodwin et al. (26) or using a miniprep procedure developed for *Pythium* spp. (35). DNA concentrations were determined spectrophotometrically or by quantitation on agarose gels stained with ethidium bromide in comparison with commercially obtained standards. A boiling miniprep procedure (36) also was evaluated using mycelium that had been grown on half-strength V8 broth (34) for 5 days or aerial hyphae collected from the surface of a potato dextrose agar culture. A small amount of hyphae was placed in 400 µl of 10 mM Tris buffer (pH 8.0) and placed in a boiling water bath for 5 min. After finger vortexing to mix the contents, the mycelium was pelleted by a brief centrifugation and 1 to 2 µl of the supernatant was added to the PCR mix.

DNA amplification and RFLP analysis. Templates were amplified by PCR using primers previously described for amplification of the *cox*I and II gene cluster for sequencing (37). FM 75 (dCCTTGGCAATTAGGATTTCAAGAT) was used for the forward primer and a mixture of FM 77 (dCACCAATAAAGAATAACCAAAAATG) and FM 83 (dCTCCAATAAAAAATAACCAAAAATG) was used for the reverse primers. While FM 75 and FM 77 amplify most species, *P. capsici*, *P. cinnamomi*, *P. citricola*, and *P. colocasiae* were amplified better using FM 83 instead of FM 77. Amplification reactions were done in 50 µl and contained approximately 20 to 50 ng of DNA, a final concentration of 1 µM forward and reverse primers, 5 µl of 10× buffer, 100 µM each dNTP, 3 mM $MgCl_2$, and 3 units of *Taq* DNA polymerase (Promega, Madison, WI). Amplifications were done using an Eppendorf Mastercycler Gradient Thermalcycler (Eppendorf Scientific, Westbury, NY) with a 3°C/s ramping time using the following parameters: one cycle at 95°C for 3 min; 35 cycles of 1 min annealing at 60°C, 1-min extension at 72°C, and 1-min denaturation at 94°C; followed by one extension cycle at 72°C for 10 min. Prior to digesting amplicons, all amplifications were separated on a 1.5% agarose gel to check DNA concentration and purity.

Digestions with restriction enzymes were conducted overnight in a total volume of 17 µl in accordance with the manufacturer's recommendations (New England Biolabs, Beverly, MA) and were separated in 3% NuSieve 3:1 agarose (Cambrex Bio Science, Rockland, ME) in 0.5× TBE buffer (0.045 M Tris-borate and 0.001 M EDTA, pH 8.0) at 45 V for 6 h or until the bromophenol blue dye in the loading buffer had migrated 8 cm from the well. A 100-bp ladder (New England Biolabs) mixed with a nondigested amplicon from *P. infestans* (isolate 580; approximately 2.2 kb based on sequence analysis) was used as size markers. The gel was stained in ethidium bromide (0.5 µg/ml) for 30 min, destained in deionized water for 30 min, and photographed under short wave UV using either Polaroid Type 55 film or an ERDAS imaging system (Kodak, Rochester, NY).

Data analysis. Scanned digital images of the Polaroid negatives or the digital images from the ERDAS imaging system were imported into the computer program BioNumerics (version 2.5, Applied Maths, Sint-Martens-Latem, Belgium). This computer program automatically determines the molecular size of the RFLP bands relative to molecular size standards included on each agarose gel. The images were processed using standard procedures and molecular size determinations of digested bands done automatically with manual confirmation. To optimize band matching between isolates, the positional tolerance of each band (the maximum shift between two bands that is allowed to consider the bands matching, expressed as a percentage of the length of the gel run) and optimization of the data (this allows for a shift between any two patterns to optimize comparisons of banding patterns, expressed as a percentage of the run length) was determined from the data using the tolerance and optimization options of the program. Positional tolerances of 1.06, 1.0, 1.16, and 1.0% were obtained for *Alu*I, *Msp*I, *Rsa*I, and *Taq*I, respectively. Optimization was determined to be 0 except for *Rsa*I, which was set at 0.54%. Bands less than 100 bp in size were excluded from the analysis due to the diffuse nature of the bands when using a 3% NuSieve 3:1 agarose gel. In addition to band size determinations, BioNumerics also has database management and analytical capabilities for data analysis. One of the available functions is the ability to perform cluster analysis to evaluate the similarity in restriction banding profiles among the various species evaluated.

RESULTS

Species identification based on RFLP patterns. Using the amplification conditions noted, primers FM 75 and FM 77/83 amplified target sequences from all *Phytophthora* spp. investigated, including those where DNA was extracted using the boiling miniprep procedure (data not shown). In cases where the template DNA from the boiling miniprep method did not amplify, dilution of the DNA with water prior to adding to the PCR amplification mixture generally allowed amplification. Following digestion with *Alu*I, an RFLP pattern was observed that in many cases was species-specific and had low levels of intraspecific variation (Fig. 1; Table 2). Depending on the restriction digest, faint bands in addition to the more intensely staining bands were seen in some lanes; because they were not consistently present in different digests (Fig. 1A to C), they were believed to represent partial digests and were not included in the analysis. To identify additional polymorphisms that would increase the resolving power of species identification, the amplicons were individually digested with a total of four restriction enzymes (Table 2). The resulting banding patterns were grouped by unweighted pair group method using arithmetic averages (UPGMA) analysis of Jaccard's similarity coefficients using the computer program BioNumerics (Fig. 2). Since bands below 100 bp were not included in the analysis and doublet bands were scored only once, summation of band sizes for individual restriction enzymes in Table 2 did not always add up to the same total size for each isolate.

For most species, no intraspecific variation in banding patterns was observed (*P. cinnamomi*–4 isolates, *P. colocasiae*–6 isolates, *P. cryptogea*–2 isolates, *P. drechsleri*–3 isolates, *P. fragariae* var. *fragariae*–5 isolates, *P. hibernalis*–4 isolates, *P. ilicis*–3 isolates, *P. infestans*–11 isolates, *P. lateralis*–3 isolates, *P. mirabilis*–4 isolates, *P. nemorosa*–2 isolates, *P. nicotianae*–12 isolates, *P. pseudosyringae*–6 isolates, *P. phaseoli*–6 isolates, *P. ramorum*–24 isolates, and *P. sojae*–3 isolates). For other species, limited variation was present, which could be explained by the addition/loss of a single restriction site. For example, *P. cactorum* isolate 385 was identical to the other seven isolates of *P. cactorum* with the exception of an additional *Msp*I site. Likewise, *P. citricola* isolate Cr-4 differed from the other two isolates by an additional *Alu*I site, and *P. palmivora* isolate 329 and Pl-10 differed from isolates Pl-5 and Pl-14 by an additional *Msp*I site. *P. erythroseptica* isolate 368 had an identical banding profile as two isolates of *P. cryptogea* and differed from the other eight isolates of *P. erythroseptica* by an additional *Alu*I site. For *P. capsici*, the results were more variable; seven isolates recovered from vegetable crops were identical and differed from an additional three isolates from vegetables (Cp-30, Cp-32, and 307) by the absence of an *Msp*I site. One additional isolate, Cp-1 isolated from *Theobroma cacao*, had identical *Alu*I and *Rsa*I RFLP profiles as the other isolates, but had a different *Msp*I site than Cp-30, Cp-32, and 307 and a different *Taq*I site than all the other *P. capsici* isolates. For some species, a higher level of intraspecific polymorphism was encountered. While the three isolates of *P. syringae* shared some similarities in banding

profiles (31.6 to 44%), the level of polymorphism observed could not be explained by single restriction site differences. The two isolates of *P. megakarya* from Nigeria and Cameroon also were polymorphic and shared only 33.3% similarity in restriction banding profiles. The greatest level of intraspecific variation observed was for the four isolates of *P. megasperma*. While some isolates grouped together on the same clade (isolates 309, 335, and 437), the restriction profiles of these isolates shared little similarity (24 to 34%).

DISCUSSION

The PCR primers previously reported for amplification of the *cox*I and II genes (37,38) amplified target DNA sequences from all 152 isolates of the 31 *Phytophthora* spp. evaluated in this study. Amplified fragments digested with four restriction enzymes provided restriction-banding profiles that identified isolates to a species level. While digests with *Alu*I alone could generate a species-specific diagnostic banding profile able to differentiate most species evaluated in this investigation, a total of four restriction enzymes were used to increase the level of variation observed among species and improve the resolution of the technique. Using this approach, 16 of 24 species in which multiple isolates were examined did not exhibit polymorphisms in their restriction profiles. While no intraspecific variation was observed among the two isolates of *P. cryptogea* or three isolates of *P. drechsleri* examined in this study, given the polyphyletic origin of these species based on isozymes, mitochondrial DNA (mtDNA) RFLPs, and ITS1 sequence analysis, (21,42) analysis of additional isolates representing the diversity of the species is needed to clarify the utility of this RFLP marker system for identification of these species.

The intraspecific polymorphisms observed in five additional species (*P. cactorum*, *P. capsici*, *P. citricola*, *P. erythroseptica*, and *P. palmivora*) could be explained by the presence/absence of a single restriction site. The only exception to this was *P. capsici* isolate Cp-1 from *Theobroma cacao*, which had a single addition/loss of a restriction site for both *Msp*I and *Taq*I. This observation may be reflective of the different groupings observed for the same tropical isolate from *T. cacao* and some of the temperate isolates recovered from vegetable crops in a *cox*II phylogeny and for amplified fragment length polymorphism banding profiles (5). Grouping of isolates based on isozymes (41,46) and mtDNA RFLP analysis (23,30) has been reported, although a clear distinction between host or tropical/temperate location of recovery was not observed. For example, Förster et al. (23) observed groupings based on mtDNA RFLPs that did not clearly delineate between hosts or location of recovery, while Hwang et al. (30) observed four groupings of isolates from pepper based on mtDNA RFLPs. Likewise, Mchau and Coffey (41) observed two major groupings of *P. capsici* isolates based on analysis of 15 isozymes that each included isolates from a range of hosts and geographic locations. Aragaki and Uchida (1) have proposed classifying isolates from one of these groups (tropical hosts that are avirulent on pepper and produce chlamydospores and elongate sporangia) in the separate species *P. tropicalis*. Clearly, additional isolates of *P. capsici* and *P. tropicalis* recovered from a wider range of hosts and geographic locations need to be evaluated to fully assess the utility of the mtDNA PCR-RFLP procedure for differentiation of these species.

A greater level of intraspecific polymorphism in RFLP profiles was observed for *P. megakarya*, *P. syringae*, and *P. megasperma*, reflecting the variation that has been reported in the literature among isolates of these species. Many isolates for these three species used in this study have been included in phylogenetic analyses based on DNA sequence data of the mitochondrially encoded *cox*II gene (37,38). The two isolates of *P. megakarya* from Nigeria and Cameroon had a 33.3% similarity in the RFLP banding profile and exhibited 3.4% sequence divergence for the *cox*II gene, which compared to a divergence of generally less than 0.5% for intraspecific comparisons among most of the other *Phytophthora* spp. examined (37). A distinct grouping of these same isolates of *P. megakarya* were also reported by Förster et al. (23) using RFLP analysis of the entire mitochondrial genome, with a similarity of approximately 55% observed between them. The three isolates of *P. syringae* evaluated in this investigation exhibited a 31.6 to 44% similarity in RFLP banding profiles, with isolate 442 grouping with 468. A similar grouping was observed in the *cox*II gene analysis with a sequence divergence ranging from 0.3% between isolates 442 and 468, and 3.6% for these two isolates and isolate 469 (38).

Likewise, the four isolates of *P. megasperma* that were examined exhibited 24 to 34% similarity in RFLP profiles and a *cox*II

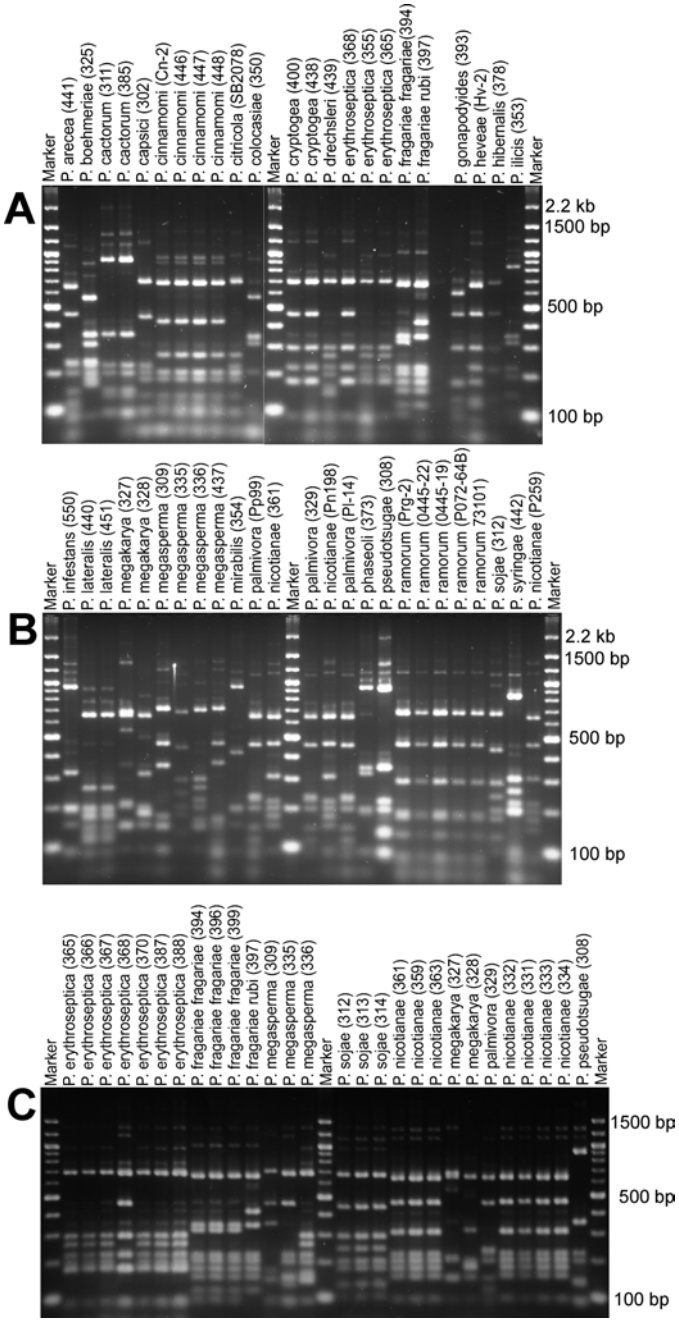

Fig. 1. The polymerase chain reaction-amplified fragment using primers FM 75 and FM 77/83 was digested with *Alu*I and separated in a 3% NuSieve 3:1 agarose gel. The size marker is a 100-bp ladder. In **A and B** an undigested amplicon from *Phytophthora infestans* (2.2 kb) was added to the size marker well.

sequence divergence of up to 4.9% (37). Variability among isolates of this species has been well documented based on both mitochondrial and nuclear DNA analysis. Using mtDNA RFLP analysis, Förster et al. (23) observed several subgroupings among isolates classified as *P. megasperma*, indicating a polyphyletic origin for the species complex. In a subsequent study evaluating 194 isolates of *P. megasperma*, Förster et al. (20) observed nine distinct molecular groupings. Three of these groupings are currently classified as *P. medicaginis*, *P. sojae*, and *P. trifolii* (18), two groups are host specific and commonly associated with asparagus or Douglas fir, and the remaining four groups were broad host range isolates from woody and herbaceous hosts. Isolates within these groupings were also variable, with similarity values as low as 49%. Furthermore, there were an additional 20 isolates with unique RFLP patterns that were not associated with the above nine distinct molecular groupings. The isolates used in this current study, IMI133317, 335, 309, and 336, were examined by Förster and Coffey (20) and grouped in mtDNA group A, D, C, and F, respectively, which exhibited a range of 19 to 41% similarity in mtDNA banding profiles among them. With the exception of isolates IMI133317 and 309, the isolates also varied in protein grouping, colony type, and other characteristics (29). Likewise, isolate 339 was classified as a Douglas fir group 2 isolate that differed in virulence and host range from Douglas fir group 1 isolate 336 (28). Comparisons of *P. megasperma* isolates based on ITS sequence analysis confirmed the polyphyletic nature of this species complex (6,21). Additional studies including the analysis of the ITS region or another nuclear encoded region for isolates of *P. megakarya*, *P. syringae*, and *P. megasperma* are needed to clarify the mtDNA PCR-RFLP results obtained.

While some groupings of species on specific clades observed in Figure 2 were also seen on the phylogenetic trees obtained using sequence analysis of the mitochondrially encoded *cox*II gene (37,38), this was observed only in a general sense and was not the case for all species. Therefore, the relationships among species in the RFLP phenogram (Fig. 2) should be viewed in the context of isolate identification only and not for inferring phylogenetic relationships within the genus. The grouping of *P. erythroseptica* isolate 368 with the two *P. cryptogea* isolates in Figure 2 was also observed in the *cox*II phylogenetic study (37) and warrants further examination of additional isolates of these species to clarify their relationship. A similar close grouping however was also observed for some members of these two species based on ITS1 sequence alignments (21). The close grouping of *P. arecae* with *P. palmivora* agrees with results obtained in the *cox*II phylo-

TABLE 2. Sizes of restriction fragments for *Phytophthora* spp. amplified with primers FM 75 and FM 77/83 and digested with the indicated restriction enzyme[a]

Phytophthora sp.	Isolate no.	*Alu*I	*Msp*I	*Rsa*I	*Taq*I
P. arecae	441	648, 460, 238, 227, 203, 143, 117	2190	663, 550, 332, 300, 226, 182	2184
P. boehmeriae	325	565, 355, 315, 238, 227, 201, 179, 164	1305, 1016	621, 550, 332, 290, 182	1870, 437
P. cactorum	311	944, 327, 227, 201, 153, 132	1145	684, 550, 332, 300, 182, 148	2184
	385	944, 327, 227, 201, 153, 132	1145, 564	684, 550, 332, 300, 182, 148	2184
P. capsici	302	697, 451, 227, 203, 179, 132	2190	712, 550, 332, 300, 226, 182	1573, 743
	307	697, 451, 227, 203, 179, 132	1415, 901	712, 550, 332, 300, 226, 182	1573, 743
	Cp-1	697, 451, 227, 203, 179, 132	1140, 1080	712, 550, 332, 300, 226, 182	1187, 834
P. cinnamomi	448	697, 421, 267, 202, 179, 132	1145, 755, 412	712, 586, 405, 332, 300	2004, 319
P. citricola	SB2078	697, 267, 227, 203, 179, 132	2190	783, 586, 332, 300, 182, 148	2184
	Cr-4	697, 289, 267, 227, 203, 179, 132	2190	783, 586, 332, 300, 182, 148	2184
P. citrophthora	414	697, 267, 227, 203, 179, 132	2190	712, 550, 332, 300, 226, 182	2184
P. colocasiae	350	565, 354, 315, 203	1533, 532, 218	712, 550, 332, 290, 226, 182	1870, 457
P. cryptogea	438	697, 466, 298, 227, 179	1679, 564	712, 550, 332, 300, 226, 182	1619, 743
P. drechsleri	439	697, 298, 227, 201, 153, 143	1333, 564, 364	712, 550, 332, 300, 226, 182	2004, 373
P. erythroseptica	368	697, 466, 298, 227, 179	1679, 564	712, 550, 332, 300, 226, 182	1619, 743
	365	697, 298, 267, 245, 227, 201	1679, 564	712, 550, 332, 300, 226, 182	1619, 743
P. fragariae var. *fragariae*	393	665, 354, 327, 227, 203, 179, 153, 117	1145, 673, 490	712, 332, 290, 262, 226, 178, 125	2184
P. fragariae var. *rubi*	397	665, 421, 354, 227, 203, 179, 153, 117	1533, 603, 155	684, 332, 290, 262, 226, 178, 125	2184
P. gonapodyides	393	697, 466, 227, 203, 153	2101, 211	684, 550, 332, 300, 116	1278, 591, 437
P. heveae	Hv-2	665, 466, 298, 227, 179, 132, 117	2190	712, 550, 332, 300, 226, 182	1801, 284, 205
P. hibernalis	378	697, 466, 298, 227, 143	1533, 445, 327	621, 550, 332, 299, 182, 148	1120, 825, 373
P. ilicis	353	851, 354, 327, 227, 201, 143	1679, 673	663, 550, 332, 300, 182, 153	2004, 278
P. infestans	550	944, 327, 187, 153	2190	684, 351, 332, 300, 243, 226, 178	1503, 591, 205
P. lateralis	440	666, 267, 201, 179, 143, 117	1533, 755	621, 550, 332, 170, 134, 125	914, 743, 373, 205
P. megasperma	336	697, 298, 267, 227, 201, 164, 143	1969, 211, 122	712, 550, 332, 300, 226, 182	2184
	309	697, 466, 354, 179, 143	1016, 564, 445, 327	684, 550, 332, 300, 182, 153	1870, 392
	437	697, 466, 370, 164	1016, 755, 532	823, 550, 332, 300, 182	1278, 591, 392
	335	697, 451, 298, 250, 209, 179, 132	1533, 532, 211	663, 550, 332, 300, 182	1870, 437
P. megakarya	327	697, 665, 209, 153	1071, 673, 564	663, 550, 405, 332, 300, 148	2004, 548, 205
	328	648, 327, 201, 187, 153	1785, 532	663, 550, 332, 300, 226, 182	2004, 550, 205
P. mirabilis	354	944, 421, 201, 153	2190	684, 351, 332, 300, 226, 182	1703, 591
P. nemorosa	P-13	962, 356, 229, 202, 178, 149	2190	648, 553, 332, 226, 181	2184
P. nicotianae	361	648, 466, 315, 297, 203, 179, 153	2190	684, 550, 332, 226, 170, 125	2184
P. palmivora	329	648, 466, 238, 227, 201, 143, 117	1679, 564	663, 550, 332, 300, 226, 182	2184
	Pl-14	648, 466, 238, 227, 201, 143, 117	2190	663, 550, 332, 300, 226, 182	2184
P. phaseoli	373	944, 327, 315, 201	1305, 532, 490	684, 351, 332, 300, 243, 226, 182	1703, 591
P. pseudosyringae	470	944, 327, 250, 238, 209, 179	2190	823, 550, 332, 300, 182	2184
P. pseudotsugae	308	944, 354, 227, 201, 132	2190	684, 550, 332, 300, 226, 182	2184
P. sojae	312	697, 451, 298, 250, 203, 179, 132	2190	684, 550, 406, 332, 300	2184
P. ramorum		697, 466, 298, 187, 132	1305, 954	621, 550, 332, 226, 178, 125	2184
P. syringae	442	852, 315, 267, 227, 203	2190	1101, 550, 332, 300	1187, 1120
	468	852, 315, 267, 227, 210, 152	2190	1101, 550, 332, 300	1187, 1019, 113
	469	944, 327, 250, 209, 179	2190	762, 550, 332, 300	1187, 1120

[a] Amplified products were digested with the indicated restriction enzyme and separated in a 3% NuSieve 3:1 agarose gel. Fragment sizes were determined, and the database was managed using the computer program BioNumerics (version 2.5). Although fragment sizes are reported to the base pair by BioNumerics, this level of accuracy is artificial and not supported by the agarose electrophoresis method used for estimation. Doublet bands and fragment sizes smaller than 100 bp are not reported or included in the cluster analysis.

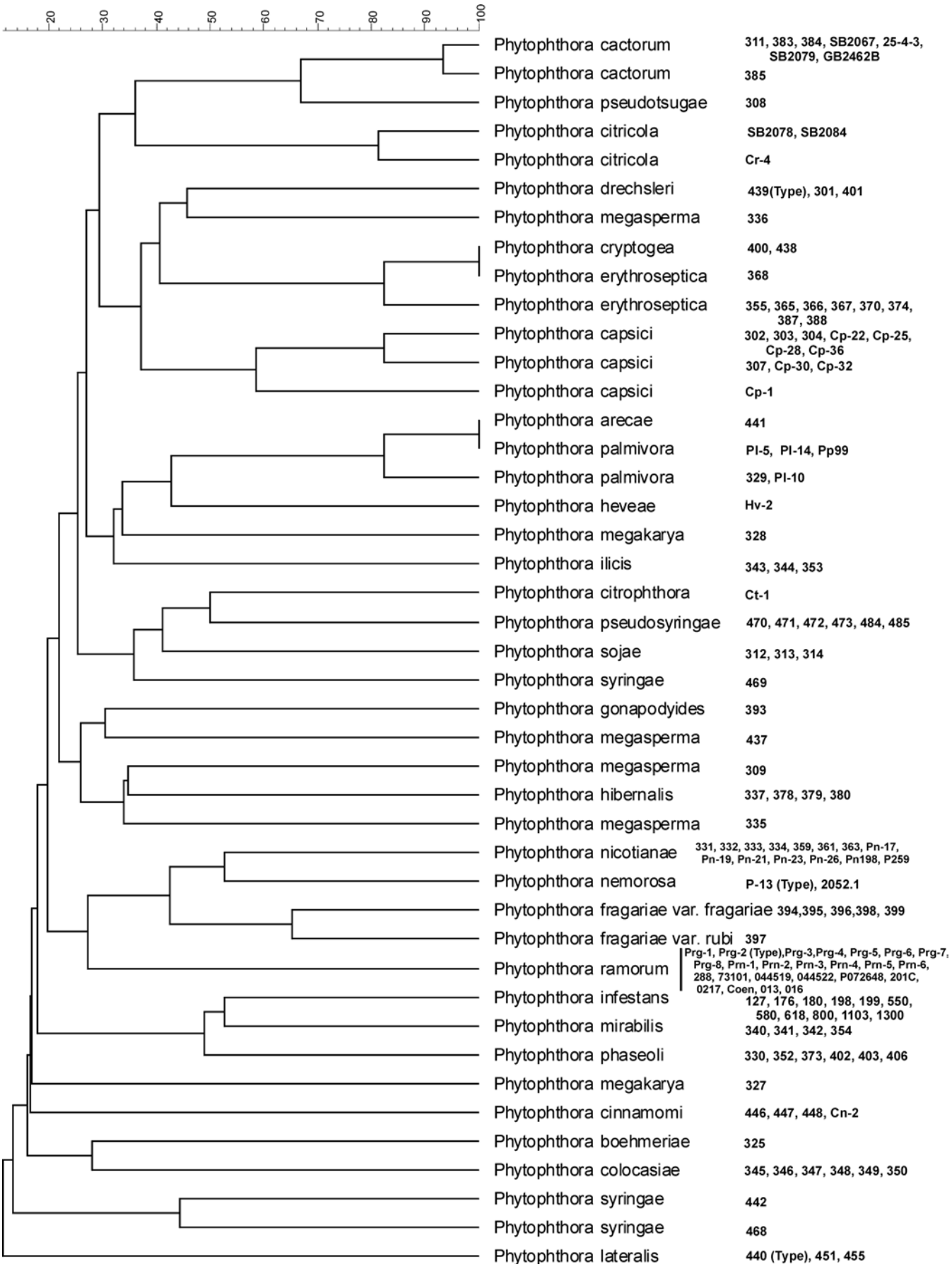

Fig. 2. Unweighted pair-group method with arithmetic average cluster analysis using Jaccard's similarity coefficients of the *Alu*I, *Msp*I, *Rsa*I, and *Taq*I restriction fragment length polymorphism restriction profiles of the polymerase chain reaction-amplified product generated using primers FM 75 and FM 77/83. The scale at the top represents percent similarity.

genetic study (37) and supports the conclusion that these two species are conspecific (18).

RFLP analysis of PCR-amplified ITS regions has been used previously for discrimination of *Phytophthora* species (10,12,48, 52); however, the ITS region had limited resolving power for some closely related species. For example, Tooley et al. (52) were unable to discriminate *P. infestans*, *P. mirabilis*, and *P. phaseoli* by digestion of the ITS2 region with six different restriction enzymes. Using an amplicon spanning the ITS1 and ITS2 regions, Ristaino et al. (48) were unable to discriminate *P. infestans* from *P. mirabilis* or *P. cryptogea* from *P. erythroseptica*. Likewise, Cooke and Duncan (12) were not able to differentiate between *P. cryptogea* and *P. drechsleri* or *P. fragariae* var. *fragariae* and *P. fragariae* var. *rubi*. In these examples, the mtDNA PCR-RFLPs were able to clearly differentiate these species (Fig. 2). One potential complication using the ITS-RFLP analysis for isolate identification that has not been observed thus far with the mtDNA PCR-RFLP analysis is the possibility of multiple forms of the target sequences present in single isolates. Cooke and Duncan (12) observed that the summation of band sizes for some isolates was greater than the initial amplicons size, indicating at least two forms of the ITS region were present (this was supported by sequence analysis); similar results have been reported by Brasier et al. (6). This also has been observed in the related genus *Pythium* (35; F. N. Martin, *unpublished data*).

PCR assays that generate species-specific amplicons are now being employed for detection and identification of *Phytophthora* spp. such as *P. infestans* and *P. ramorum* (14,24,39,51). A potential limitation of some of these assays is that only a species-specific amplicon is generated; if another *Phytophthora* sp. is present, the assay will not generate a positive PCR response. A recently described PCR-based detection system based on the spacer region between the *cox*I and II genes (39) employs a set of *Phytophthora* genus-specific primers for a first round amplification to assess if a *Phytophthora* sp. is present in the infected plant material. This first amplification is followed by a second amplification using a set of nested species-specific primers for identification of specific species such as *P. ramorum*, *P. nemorosa*, or *P. pseudosyringae*. In cases where the species-specific nested primers do not generate a PCR product, the described mtDNA PCR-RFLP analysis will be useful for identification of the *Phytophthora* sp. that is present.

One potential shortcoming in using an mtDNA-based system for identification of isolates of *Phytophthora* to a species level is that interspecific hybrids of *Phytophthora* spp. have been identified (4,7,15,16,27,33). In crosses between opposite mating types of *P. nicotianae* (*P. parasitica*), the mitochondrial genome was uniparentally inherited (19), and if this also occurs with other species in the genus, the hybrids would have a single mitochondrial genotype representative of one of the parents. Depending on which species functioned as the maternal parent and contributed the mitochondria, the use of the described RFLP marker system will likely generate a restriction profile for only one of the parental species when in fact the isolate represented a hybrid. This was observed in natural hybrids of *P. nicotianae* and *P. cactorum*, all of which had the mtDNA RFLP of *P. nicotianae* (33). How common an occurrence this would be for specific species or with field isolates has yet to be determined.

The described mtDNA PCR-RFLP method will be useful as an alternative, time-effective manner of accurately identifying *Phytophthora* species. Even though culturing of the pathogen for DNA extraction is necessary with this method, it represents a far more rapid means of identification compared with morphological techniques alone, in which days or weeks may be required for production of diagnostic asexual and/or sexual structures. Since a boiling miniprep procedure can provide suitable template DNA for amplification, samples can be processed as soon as aerial hyphae are present. The use of a computer program such as BioNumerics to automate the estimation of band sizes and create a database of banding profiles for a range of species also simplifies the use of this technique for isolate identification. This program also allows for the establishment of a library of known isolates that can be queried with the RFLP patterns of unknown isolates to provide a statistical breakdown of the similarity in banding profiles among isolates.

The utility of the PCR-RFLP system would be improved if amplification could be performed directly from infected tissue, thereby bypassing the need for pathogen culturing. While the primers FM 75 and FM 77/83 do not amplify bands from a range of different plant species, they do amplify bands from the related genus *Pythium* (F. N. Martin, *unpublished data*). Due to the near ubiquitous nature of *Pythium* spp. in soil, this may complicate using RFLP banding patterns of amplicons generated from infected root tissue as a taxonomic aid due to the multiple banding patterns that would be observed in co-infected plants. Attempts to directly amplify *Phytophthora* DNA from infected plant material used in a study on *Phytophthora* spp. associated with diseased forest trees (39) using primers FM 75 and FM 7/83 thus far has not been successful, presumably due to the low concentrations of template DNA compared with host DNA (F. N. Martin, *unpublished data*). However, this may be a technical problem that could be resolved with the development of nested primers to use in a second round of PCR amplification, the product of which would then be used for RFLP analysis. Such studies are currently in progress.

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